PEPTIDE FRAGMENT OF RESPIRATORY SYNCYTIAL VIRUS PROTEIN G, IMMUNOGENIC AGENT, PHARMACEUTICAL COMPOSITION CONTAINING IT AND PREPARATION PROCESS

The present invention relates to polypeptides which can be used especially in the preparation of immunogens and the obtainment of vaccine against respiratory syncytial virus (RSV) and to nucleotide sequences enabling them to be obtained. The invention likewise relates to an immune adjuvant protein extracted from Klebsiella pneumoniae, to compositions comprising the immunogenic polypeptides, possibly associated with such an adjuvant protein, and to their preparation process.

Respiratory syncytial virus (RSV) is the most frequent cause of respiratory illnesses in the newborn: bronchopneumopathies (bronchiolites). The WHO estimates each year 50 million cases of RSV attacks, from which 160,000 die in the entire world. There are two subgroups of the virus (subgroups A and B).

RSV is classified in the Paramyxoviridae family, a type of pneumovirus comprising a nonsegmented RNA genome, of negative polarity, coding for 10 specific proteins.

There is at present no vaccine available against RSV. Inactivated virus vaccines have been shown to be inefficaceous and have sometimes even aggravated the infections of nursing infants. In the 60's, vaccination attempts with formalin-inactivated RSV resulted in failure: instead of conferring protection at the time of reinfection due to RSV, the vaccine had the effect of aggravating the illness in the child.

The Application WO 87/04185 proposed to use structural proteins of RSV with a view to a vaccine, such as the envelop[e] proteins called protein F (fusion protein) or protein G, a 22 Kd glycoprotein, a 9.5 Kd protein, or the major capsid protein (protein N).

The Application WO 89/02935 describes the protective properties of the entire protein F of RSV, possibly modified in monomeric or deacetylated form.

A series of fragments of protein F have been cloned with a view to investigating their neutralizing properties.

However, the immune vaccines tested to date have been shown to be inefficaceous or have induced a pulmonary pathology (bronchiolitis or peribronchitis).

At the present time, there is no in-depth treatment of infections due to RSV.

Infections [lacuna] to RSV of the upper airways: treatment relies essentially on symptomatic medications identical to those for other viral infections.

Infections [lacuna] to RSV of the lower airways: treatment in nursing infants relies on the maintenance of correct hydration, the aspiration of the secretions and the administration of oxygen if necessary. A positive effect has been observed with ribavirin, a nucleotide which is active in vitro against RSV.

It is for these reasons that an object of the present invention is a polypeptide which is useful especially in immunogen production, characterized in that it is carried by the peptide sequence between the amino acid residues 130 and 230 of the sequence of respiratory syncytial virus protein G, or by a sequence having at least 80% homology with said peptide sequence. This sequence differs slightly for the subgroups A and B of human RSV, or for bovine RSV. The invention comprises the sequences originating from human RSV subgroup A and B, or bovine RSV.

Protein G is an RSV envelope glycoprotein, of molecular weight of between 84 and 90 Kd, which is low in methionine.

The Applicant has demonstrated that the sequence between amino acids 130 and 230 of natural protein G is particularly appropriate for inducing an efficaceous protection against infection by RSV. The invention comprises the sequences originating from human RSV subgroup A or B, or bovine RSV.

More particularly, the present invention relates to polypeptides, which are useful especially as

immunogenic element included in the above and which comprise the peptide sequence between the amino acid residu s numbered 174 and 187 of RSV protein G (human, subgroups A and B, or bovine) or a sequ nce having at least 80% homology with the corresponding sequence.

Other peptide sequences adapted to the preparation of an immunogen included in said sequence of RSV protein G are formed by the sequence between the amino acid residues numbered 171 and 187 of human or bovine RSV protein G, or a sequence having at least 80% homology with the corresponding sequence. Other peptides of interest according to the present invention are carried by the sequence between the nucleotides numbered 158 and 190 of RSV protein G or a sequence having at least 80% homology with the corresponding sequence.

According to another method of carrying it out, the invention relates to peptides useful for the preparation of an immunogen and which have a sequence corresponding to the sequence between the amino acid residues numbered 140 and 200 of human or bovine RSV protein G, or a sequence having at least 80% homology with the corresponding sequence. Sequences starting with amino acid 140 of said RSV protein G and whose C-terminal end corresponds respectively to the amino acid 198, 196, 194, 192 or 190, as well as sequences having at least 80% homology with the sequence carried by these fragments, are particularly advantageous.

Among the variants of the above sequences, polypeptides may be mentioned which comprise a sequence in which:

a) the Cys amino acid in positions 173 and/or 186 has been replaced by an amino acid not forming a disulfide bridge, in particular serine, and/or

b) the amino acids in positions 176 and 182 are capable of forming a covalent bridge other than a disulfide bridge, especially aspartic acid and ornithine.

Thus the polypeptide sequence 130-230 of RSV subgroup A can be used complete, in its native form. This sequence corresponds to the written sequence Seq id No.

1 (or G2A).

In the same way, it is possible to use the complete polypeptide sequence 130-230 of RSV subgroup B in its native form. This sequence corresponds to the written sequence Seq id No. 2 (G2B).

The sequence id No. 1 will be written G2A in the remainder of the application.

The sequence id No. 2 will be written G2B in the remainder of the application.

Sequences having at least 80% homology with G2A or G2B are also appropriate.

The sequence between the amino acids 130 and 230 can be modified by the replacement of the cysteine residue in positions 173 and 186 by serine residues to obtain a peptide retaining good immunogenic properties, owing to maintenance of the loop formed by the Cys residues in positions 176 and 182. The amino acid and nucleotide sequences of this polypeptide for subgroup A are represented in seq id No. 3 (G2AδCys).

For subgroup B, the amino acid and nucleotide sequences are represented in seq id No. 4 (G2BδCys).

The peptide sequences will be written G2AδCys and G2BδCys.

According to another aspect, an object of the invention is a polypeptide which is useful for the preparation of immunogen, characterized in that it consists in the peptide sequence between the amino acid residues numbered 174 and 187 of RSV protein G or a sequence having at least 80% homology with said peptide sequence.

In this last sequence the peptide 174-187 subgroup A can have the sequence:

Seq id No. 5:

Ser Ile Cys Ser Asn Asn Pro Thr Cys Trp Ala Ile Cys Lys.

The peptide 174-187 subgroup B can have the sequence:

Seq id No. 6:

Ser-Ile-Cys-Gly-Asn-Asn-Gln-Leu-Cys-Lys-Ser-Ile-Cys-Lys.

The Cys residue in position 186 can also be

replaced by a serine residue, so as to obtain the following sequence:

Seq id No. 7 for subgroup A:

Ser Ile Cys Ser Asn Asn Pro Thr Cys Trp Ala Ile Ser Lys.

Seq id No. 8 for subgroup B:

Ser-Ile-Cys-Gly-Asn-Asn-Gln-Leu-Cys-Lys-Ser-Ile-Ser-Lys.

In the sequence between residues 174 and 187 of the immunogenic peptide, according to one of the variants of the invention, the amino acid residues in positions 176 and 182 are respectively replaced by an aspartic acid and an ornithine, so as to obtain one of the following sequences:

Seq id No. 9 for subgroup A:

Ser Ile Asp Ser Asn Asn Pro Thr Orn Trp Ala Ile Cys Lys

Seq id No. 10 for subgroup B

Ser-Ile-Asp-Gly-Asn-Asn-Gln-Leu-Orn-Lys-Ser-Ile-Cys-Lys.

Seq id No. 11 for subgroup A:

Ser Ile Asp Ser Asn Asn Pro Thr Orn Trp Ala Ile Ser Lys.

Seq id No. 12 for subgroup B:

Ser-Ile-Asp-Gly-Asn-Asn-Gln-Leu-Orn-Lys-Ser-Ile-Ser-Lys.

The maintenance of the immunogenic properties is obtained owing to the replacement of the disulfide bridge (between the natural Cys residues) by an amide bridge between the positions 176 and 182.

Other sequences according to the invention such as defined above appear in the annex of the present application under the names SEQ ID No. 14 to SEQ ID No. 73.

An object of the invention is likewise a polypeptide which can be used as an immunogenic agent having one of the preceding sequences and which additionally comprises at least one cysteine residue in the N-terminal or C-terminal position.

The invention likewise comprises a polypeptide which consists of the peptide sequence between the amino acid r sidues numbered 130 and 230 of the RSV protein G sequence subgroup A and subgroup B, or of a sequence having 88% homology with said peptide sequence and which is in the rorm of a fusion protein with the receptor of

human serum albumin, called BBG2AδC or BBG2BδC, or another linking protein. The sequence of the complete BB protein appears in the annex (Seq ID No. 74).

The invention likewise comprises the variants, for example glycosylated or sulfated, of the different peptides, whether these functions are natural or not.

The polypeptides can be prepared by peptide synthesis or by recombinant DNA techniques, which are known to the person skilled in the art.

In particular, the gene sequences coding for the epitope of approximately 100 amino acids can be prepared by solid-phase assembly of genes, and the corresponding protein expressed, for example, in E. coli by the intracellular route.

The nucleotide sequences (RNA or DNA) coding for the proteins or the polypeptides defined above are part of the invention.

Another object of the invention is an immunogenic agent which comprises a polypeptide such as defined above coupled to a carrier protein, in particular to an immune adjuvant protein.

Preferably, the polypeptide according to the invention is coupled to a carrier protein of the type OmpA of the external membrane of a bacterium of the genus Klebsiella, preferably in the form of a soluble conjugate.

The Applicant has been able to show that although the variants of the sequence 174-187 of the RSV protein G are weakly immunogenic, their coupling with such a protein induces a specific immune response.

The intensity of the immune response has been compared to that obtained with conventional adjuvants, such as coupling to the carrier KLH (keyhole limpet hemocyanin) coadministered with Freund's adjuvant, or coupling to the carrier protein TT (tetanus toxoid).

Particularly advantageous results are obtained for compositions comprising an immunogenic polypeptide according to the invention coupled to protein p40 of Klebsiella pneumoniae or a protein having 80% homology

with protein p40.

More particularly, said polypeptide is coupled to a protein comprising the peptide sequence written Seq id No. 13.

The nucleotide sequence (DNA or RNA) coding for the protein comprising the sequence id No. 13 is comprised in the invention.

The immunogenic polypeptide can be coupled to the immune adjuvant protein by methods known to the person skilled in the art, such as:

- Glutaraldehyde
- Carbodiimide (e.g.: EDC: 1-(3dimethylaminopropyl)-3-ethylcarbodiimide[sic]).
- Bis imido esters (e.g.: dimethyl adipimidate).
- N-hydroxysuccinimidyl esters (e.g.: disuccinimidyl suberate).
- For peptides comprising a supplementary cysteine in the N terminal or C terminal position:
  * Maleimido-N-hydroxysuccinimide esters (e.g.: MBS: maleimido benzoyl-N-hydroxysuccinimide ester).
  * N-succinimidyl bromoacetate.

The polypeptide can be conjugated to the carrier protein by a linking protein, for example the human serum albumin receptor (BB).

According to another aspect, an object of the invention is likewise a process for the preparation of a conjugated peptide inserted in a composition useful for prevention or treatment of infections with RSV, characterized in that:

a) the membrane lipopolysaccharides of bacteria of the genus Klebsiella are precipitated in the presence of a salt of a divalent cation and of detergents to recover the total membrane proteins in the supernatant,

b) the proteins are submitted to anion-exchange chromatography to separate the fraction containing the immune adjuvant protein,

c) the fraction containing the immune adjuvant protein is concentrated,

d) the immune adjuvant protein is conjugated with an immunogenic polypeptide such as defined above to form a soluble conjugate.

The divalent cation salt used in step a) is preferably a salt of calcium or of magnesium. After centrifugation, the proteins of the supernatant can be recovered in good yield by two precipitations with ethanol.

The membrane proteins, after resuspension, are separated on an anion-exchange column which can be used under industrial conditions. This chromatographic support is very stable and compatible with drastic pyrogen removal treatments, which was not the case with the chromatographic supports already described. On the other hand, elution of the protein can be carried out under isocratic conditions and not by application of an NaCl gradient (as described previously), which is particularly advantageous under industrial conditions.

According to a preferred method of carrying out the invention, step c) is followed by a second chromatography step, on a cation exchanger, and the fractions containing the adjuvant protein are recovered and concentrated. This supplementary step allows a better elimination of the lipopolysaccharides. The adjuvant protein is then conjugated to an immunogenic polypeptide according to the invention.

According to another aspect, the invention relates to a composition useful for the prevention and/or treatment of infections provoked by RSV, characterized in that it contains a polypeptide characterized above.

More particularly, the compositions additionally contain pharmaceutically acceptable excipients adapted for administration by the injectable route.

In fact, the Applicant has demonstrated that the injection of such compositions affords protection, not by a neutralizing effect, but by a systemic immune response of the body.

The humoral and cellular responses (IgM, IgG, IgA and T cells) are provoked by the product which likewise

induces a long-term protection and an immunological memory against the RSV subgroups a and b.

With a view to the administration of the vaccine compositions by the subcutaneous route, it is desirable to have available soluble conjugate, which is difficult by the conventional methods.

It is for this reason that the invention likewise relates to a process for the preparation of a conjugate between an immunogenic peptide and a membrane protein of Klebsiella, in particular the protein p40 of K. pneumoniae, in which the coupling is carried out in the presence of glutaraldehyde at concentrations lower than or equal to 0.05%.

This coupling process considerably reduces the concentrations of glutaraldehyde in comparison with those usually used (2 times 0.01% instead of 1% approximately); the glutaraldehyde is added in 2 portions over a period of five days although the protocols described mention times of 24 hours.

These modifications have allowed the obtainment of a soluble conjugate, in a form adapted for subcutaneous administration.

The usual protocols (higher concentrations of glutaraldehyde and short times) are manifested by the formation of a thick gel (due to P40-P40 conjugation reactions, very probably), a form unfit for administration and manipulation in general.

The conjugated peptide can be frozen and used as such or lyophilized.

The examples which follow are intended to illustrate the invention without in any way limiting the range thereof.

In these examples, reference will be made to the following figures:

- Figure 1: intensity of the immune response induced against G1A in different forms,
- Figure 2: kinetics of the immune r sponse induced against G1A presented in different forms,
- Figure 3: kinetics of the immune response induced

against the carrier alone,

- Figur 4: cloning strategy by genetic amplification of p40.

Example 1: Synthesis and purification of G_1A

The polypeptide of sequence

Ser-Ile-Cys-Ser-Asn-Asn-Pro-Thr-Cys-Trp-Ala-Ile-Ser-Lys

|_____ S-S _____|

written G_1A is prepared by solid-phase synthesis using Boc chemistry.

Assembly

The assembly of the peptide is carried out by solid-phase peptide synthesis on polystyrene (divinylbenzene 1%), starting with a Boc-Lys(2-cl-Z)-phenylacetamidophenyl linking agent.

The Boc-benzyl chemical strategy was used with the following deprotection-coupling procedure:

1.	55% TFA in DCM	(1 x 5 min)
2.	55% TFA in DCM	(1 x 25 min)
3.	DCM	(2 x 1 min)
4.	Isopropyl alcohol	(1 x 1 min)
5.	DMF	(2 x 1 min)
6.	10% DIEA in DMF	(2 x 2 min)
7.	Coupling	
8.	DMF	(2 x 1 min)
9	DCM	(2 x 1 min)

In each step, 20 ml of solvent are used per gram of peptide resin.

The coupling is carried out in DMF with a preformed hydroxybenzotriazole ester for 30 min. It is verified in each step of the coupling if residual free amine functions are present by the ninhydrin test. If necessary, a double coupling is carried out.

For the synthesis of the G_1A peptide, the following side-chain protection groups were used:

- 2-chlorobenzyloxycarbonyl for lysine,
- benzyl for serine and threonine,

- 4-methylbenzyl for cysteine,
- formyl for tryptophan.

Before the final deprotection/cleavage step, the formyl group is eliminated by treatment for 30 min with a 25% solution of piperidine in DMF. The peptide resin is washed with DCM and ether, and dried under reduced pressure.

Cleavage

The peptide is cleaved from the resin and completely deprotected by treatment with liquid hydrogen fluoride. 10 ml of hydrogen fluoride per gram of peptide resin are conventionally used at 0°C for 45 min in the presence of p-cresol and ethanedithiol as a trap. After evaporation of the hydrogen fluoride, the crude reaction mixture is washed with ether, dissolved in TFA, precipitated with ether and dried.

Cyclization and purification

General conditions of purification by HPLC:

Stationary phase:	C_{18} silica, 15-25 µm, 100 Å
Mobile phase:	solvent A: water 0.1% TFA
	solvent B: acetonitrile/A, 60/40% (v/v)
Linear gradient:	20 to 50% B in 30 min (first purification step)
	15 to 40% B in 30 min (second purification step)
Flow rate:	40 ml/min
Detection:	UV (210 nm)

The crude peptide obtained after cleavage is purified under the conditions described above (gradient of 20 to 50% B). Fractions having a purity of greater than 70-80% (HPLC) are combined and lyophilized. The peptide is then purified in a mixture of acetonitrile water and DMSO (1 mg/ml) and stirred until the cyclization is complete (4 to 6 days). The progress of the reaction is checked by HPLC. The reaction mixture is finally concentrated on a preparative HPLC column and a

gradient of 15 to 40% of B is applied in 30 min so as to purify the peptide.

Generally, after lyophilization, a second purification under the same condition is carried out to attain the degree of purity required.

The purity and the identity of the final product are checked by analytical HPLC, amino-acid analyzis and FAB mass spectrometric analyzis.

In the peptide thus obtained, the serine residue in position thirteen replaces the Cys residue of the natural peptide, thus avoiding heterogeneity in the formation of disulfide bridges, which can be harmful to the immunogenicity.

Example 2: Preparation of the epitope $G_2A\delta Cys$

Gene construction: materials and methods

In an Eppendorf microtube, 300 µg of beads are washed with washing/binding buffer (1M NaCl, 10mM Tris-HCl pH7.5, 1 mM EDTA) before adding 0.2 pmol of biotinylated oligonucleotide; 15 minutes' incubation at ambient temperature for binding. The beads with the immobilized oligonucleotide are rinsed and sedimented. 0.2 pmol of the following 5'-phosphorylated oligonucleotide is added in 60 µl of hybridization/ligation buffer (50mM Tris-HCl pH7.6, 10 mM $MgCl_2$, 1mM ATP, 1 mM 1,4-dithiothreitol [DTT], 5% polyethylene glycol [PEG] 8000). The hybridization mixture is incubated at 70°C for 5 min and allowed to come to 37°C before adding 3 units of T4 DNA ligase (BRL) followed by 15 min incubation at 37°C. The reaction mixture is rinsed before adding 0.2 pmol of the following oligonucleotide. The hybridization/ligation procedure is repeated as many times as a new 5'-phosphorylated complementary oligonucleotide is added. At the end, the DNA duplex immobilized on magnetic beads can be separated from the support by cutting with the appropriate restriction enzymes.

The DNA corresponding to th sequence G2AδCys and

to the sequence G2AδCys attached to the linking protein to human serum albumin (BB) written BB-G2AδCys is prepared.

The nucleotide sequence is expressed in E. coli to recover the corresponding proteins.

Expression vector:

pVABBG2AδC is an expression vector of the intracellular type, it contains a promoter of *E. coli* origin, the tryptophan (Trp) operation [sic], followed by the gene coding for the receptor of human serum albumin BB (P-Å Nygren et al., J. Mol. Recognit., 1988, 1, 60) and finally the gene coding for G2AδC of RSV. The expression of the heterologous gene can be induced in the presence of IAA (3-β-indoleacrylic acid). The fusion product BBG2AδC can be purified by affinity on an HSA-sepharose column, after having liberated the cytoplasmic proteins of *E. coli*.

Examples of purification of proteins starting from 500 ml of culture:

The strain *E. coli* RV 308 (Maurer et al., J. Mol. Biol., 1980, 139, 147) transfected by the plasmid pVABBG2AδC was selected on agar containing ampicillin (100 μg/ml) and tetracycline (8 μg/ml). The strain was inoculated into an Erlenmeyer flask containing 100 ml of TSB culture medium (Tryptic Soy broth, Difco) (30 g/l), supplemented with yeast (Yeast Extract, Difco) (5 g/l), ampicillin (100 μg/ml), tetracycline (8 μg/ml) and tryptophan (100 μg/ml). Incubate at 32°C for 12 hours with stirring (190 rpm). Transfer the culture into another erlenmeyer flask (5 liters) containing four times the initial volume (400 ml of TSB + yeast + the same antibiotics at the same concentration). When the optical density of the medium (at 550 nm) has reached an O.D. of approximately 1.5, the production of the proteins is induced by adding IAA to the medium to a final concentration of 25 μg/ml. Culturing is stopped after incubation for 5 hours, with stirring (190 rpm) at 32°C. After centrifugation, the bacterial plug is resuspended

in a vessel comprising approximately 60 ml of cold TST solution (50 mM TrisHCl, pH 8.0, 200mM NaCl, 0.05% Tween 20, 0.5 mM EDTA).

A standard sonicator probe (VIBRA-CELL, Somics Mat, USA) is introduced into the vessel. Sonication is carried out at a power of 5 for approximately two minutes. The supernatant of the solution after centrifugation is filtered at 0.45 μm, and passed into a column containing approximately 3 ml of HSA-sepharose gel (STÅHL et al., J. Immunol. Meth., 1989, 124, 43).

The purified proteins are analyzed by SDS-PAGE on a Phast System apparatus (PHARMACIA) or on Mini Protean BIORAD. The gels are visualized by Coomassie Blue. The protein BBG2AδC, representing more than 90% purity, corresponds well to the expected size (39.3 Kda) with respect to known molecular weight standards.

The immunotransfer of this protein to a Problott membrane (ABI) allows anti-BB and/or antiprotein G of RSV (ss-group A) to be identified with specific antibodies. The yield of purified soluble proteins starting from the cytoplasm of *E. coli* is approximately 50 mg/liter of culture.

In a 2-liter fermenter, it is possible to obtain 500 to 800 mg of BBG2AδC proteins per liter of culture under optimum culture conditions.

Example 3: Isolation and purification of the natural p40 protein

The process of purification of the P40 protein starting from the biomass of Klebsiella pneumoniae, strain I-145, was developed with one main objective: to develop a process allowing transposition to a large scale and industrial extrapolation. This process successively brings into play the preparation of a fraction enriched in membrane proteins and the purification of the P40 protein by chromatography.

MATERIALS AND METHODS

The biomass of Klebsiella pneumoniae (strain

I-145, 40 g of dry cells) is adjusted to pH 2.5 with the aid of pure acetic acid.

After addition of ½ volume of a solution comprising 6% cetrimide, 60% ethanol, 1.5 M CaCl2 [sic] whose pH is adjusted to 2.5 with acetic acid, the mixture is stirred for 16 hours at ambient temperature.

After centrifugation for 20 min at 15,000 g at 4°C, the proteins of the supernatant are precipitated with ethanol. Two successive precipitations with intermediate centrifugation (10 min, 10,000 g, 4°C) are carried out: from 20 to 50% then from 50 to 80%.

The plugs obtained after the second precipitation are resuspended in a solution of zwittergent 3-14, 1%.

After stirring for 4 hours at ambient temperature, the pH is adjusted to 6.5 with the aid of 1 N NaOH.

Centrifugation of the mixture for 20 min at 10,000 g at 4°C allows a fraction enriched in membrane proteins (MP fraction) to be obtained.

The proteins of the MP fraction are dialyzed against a 20 mM Tris/HCl buffer pH 8.0; zwittergent 3-14, 0.1%. The dialyzate is applied to a column containing a support of the strong anion exchanger type (column of diameter = 50 mm x H = 250 mm, Biorad Macroprep High Q gel) equilibrated in the buffer described above. The P40 protein is eluted by an NaCl concentration of 50 mM in the equilibration buffer.

The fractions containing the P40 are collected and dialyzed against a 20 mM citrate buffer pH 3.0; zwittergent 3-14, 0.1%. The dialyzate is applied to a column containing a support of the strong cation exchanger type (dimensions of the column : diameter = 25 mm x H = 160 mm, Biorad Macroprep High S gel) equilibrated in the 20 mM citrate buffer pH 3.0, zwittergent 3-14, 0.1%. The P40 protein is eluted by an NaCl concentration of 0.7 M. The fractions containing the P40 are collected and concentrat d by ultrafiltration with the aid of a Minitan Millipore tangential-flow filtration system used with membrane sh ets having a 10

kDa cutoff threshold.

RESULTS

The fractions obtained after each chromatographic step are analyzed by SDS-PAGE so as to collect those containing the P40 protein.

The quantities of proteins are measured by the method of Lowry (Table I). The purity and homogeneity of the P40 protein are estimated by SDS-PAGE, in the presence of molecular weight standards.

After the cation exchange chromatography step, the P40 protein is devoid of the major contaminant present in the MP fraction (the protein having an apparent molecular weight of 18 kDa) and has a degree of purity of greater than 95%.

The electrophoretic profile of the P40 reveals several bands. These bands are identified after immunoblot with P40 monoclonal antibodies obtained in mice. The upper major band corresponds to the denatured protein (by treatment at 100°C, 15 min in the presence of SDS), and the lower minor band to the protein in its native form.

P40 is in fact a "heat-modifiable" protein, and we have been able to verify this property with the aid of heating kinetics at 100°C in the presence of SDS. Without heating, the protein in native form has an α-helix structure which fixes more SDS and thus migrates further toward the anode than the denatured form (denaturation complete after 5 min at 100°C) which has a β-pleated sheet structure (K.B. KELLER (1978) J. Bacteriol. 134, 1181-1183).

The contamination with lipopolysaccharides (LPS) is estimated by determination by gas-phase chromatography of β-hydroxymyristic acid, the fatty acid marker of LPS of Klebsiella pneumoniae (Table I).

Table 1: Table of the quantities of protein and LPS of the fractions obtained for the different steps in the process for the purification of the p40 protein (n.d. = not determined).

	PROTEINS	YIELD	LPS
BIOMASS	40 g	-	n.d.
MP FRACTION	900 mg	2.25%	n.d.
FRACTION ENRICHED IN P40	400 mg	1%	10%
P40 PROTEIN	130 mg	0.3%	< 1%

This method is used to approximate the content of LPS in the samples from the different purification steps.

The quantity of β-hydroxymyristic acid present in the P40 fraction after cation-exchange chromatography being lower than the quantification threshold of the determination, it is possible to estimate that the quantity of residual LPS is lower than 1%.

Example 4: Cloning of the p40 protein and expression of BBp40

BACTERIAL STRAINS

* E. coli: RV 308: ATCC 31608 strain (MAURER R., MEYER B.J., PTASCHNE M., J. MOL. BIOL, 1980, 139, 147-161).

* K. pneumoniae: IP 145: C.I.B.P.F-strain

VECTORS

* pRIT 28 (Hultman et al., 1988,7: 629-638): cloning and sequencing vector containing the ampicillin resistance gene, the replication origins of E. coli and of the phage F1 as well as a portion of the lac-z gene of E. coli (β-galactosidose [sic]).

* pVABB: gene fusion expression vector.

SOLUTIONS

* Genetic amplification:

Lysis buffer:	25 mM Taps pH 9.3
	2 mM MgCl2 [sic]
Amplification buffer:	25 mM Taps pH 9.3
	2 mM MgCl2 [sic]
	tween 20 0.1%
	200 mM dNTP.

* Purification of proteins:

TST (20X):	Tris base	0.5 M
	HCl	0.3 M

	NaCl	4 M
	Tween 20	1%
	EDTA	20 mM
Washing buffer:	Tris HCl	50 mM pH 8.5
	MgCl2 [sic]	5 mM
Denaturation solution:	Gua-HCl	7.8 M
	Tris-HCl	28 mM pH 8.5
Renaturation solution:	Gua-HCl	0.5 M
	Tris-HCl	25 mM pH 8.5
	NaCl	150 mM
	Tween 20	0.05%.

MATERIAL AND METHOD

- Synthesis of oligonucleotides

The nucleotide primers were determined starting from the published part of the sequence of the OMPA of Klebsiella pneumoniae (LAWRENCE, G.J., et al., Journal of general microbiology, 1991, 137, 1911-1921) of the consensus sequence from the alignment of the sequences of 5 OMPA of enterobacteria (E. coli, S. tryphimurium [sic], S. marcescens, S. dysenteriae, E. aeroginosae [sic]), as well as sequences of peptides obtained by manual sequencing.

The oligonucleotides were synthesized according to the phosphoramidite chemical method on the "Gene Assembler Plus" apparatus from Pharmacia.

- Genetic amplification by PCR of the P40 gene

The DNA of OMPA of Klebsiella pneumoniae was amplified in the following manner.

A colony of Klebsiella pneumoniae is lysed in 10 µl of lysis buffer by heating to 95°C for 5 minutes.

1 µl of this solution serves as a source of DNA

for the amplification reactions.

These are carried out in 100 µl of amplification buffer (cf. annex), with 5 pmol of each primer and one unit of Taq polymerase enzyme (Perkin Elmer Cetus). Each cycle comprises one denaturation step of 30 seconds at 95°C followed by a hybridization of the primer to the DNA and an extension of one minute at 72°C. 30 cycles are thus carried out with the aid of a Perkin Elmer Cetus 9000 "Gen Amp PCR" thermocyclizer.

The following PCR are prepared starting from the DNA fragments amplified above.

The amplified DNA fragments are then digested, purified and ligated to the vector pRIT 28.

SEQUENCING

The fragments cloned in this way are sequenced on an Applied Biosystem 373 DNA Sequencer automatic sequencer. The sequencing reactions are carried out with the aid of the "dye Terminator" kit according to the recommendations of the supplier (Applied Biosystem) either on double-stranded DNA obtained after genetic amplification or from maxiprep or on single-stranded DNA from denatured PCR fragments (Hultman et al., Nucleid [sic] acids res.; 1989, 17:4937-4946).

EXPRESSION OF THE PROTEIN

The entire P40 gene is cloned in the expression vector pVABB. This vector allows an affinity tail "BB" to be attached to P40; B being the part of the streptococal G protein which ligates serum albumin (Nygren P.A. et al.; Journal mol. Recognit. 1988; 1, 69-74).

The strains of E. coli RV308 transformed by the vector pVABBP40 are cultured for one night at 37°C with stirring, in 100 ml of TSB supplemented with yeast extract, ampicillin (200 µg/ml) tetracycline (8 µg/ml) and tryptophan (100 µg/ml). The next day, a culture of OD = 1 for a wavelength of 580 nm is prepared in TSB +

yeast extracts + ampi + tetra.

After culturing for 10 minutes, expression of the protein is induced by addition of IAA at (25 µg/ml) to the medium. The culture is centrifuged at 4°C at 2460 g for 10 minutes.

The plug is taken up with 20 ml of TST 1 x pH 7.4, and the solution is then centrifuged at 4°C at 23,000 g for 30 minutes.

The supernatant is filtered through Sepharose which allows proteins termed soluble to be isolated. The plug is washed with washing buffer and then centrifuged at 23,000 g at 4°C for 30 minutes. The plug containing the inclusion body is then taken up with 900 µl of a denaturing solution + 100 µl of 10mM Diothiothreitol [sic] and incubated at 37°C for 2 hours.

The solution is then incubated at ambient temperature for 1 night, with stirring, [lacuna] in 100 ml of renaturation buffer at 2300 g for 1 hour.

The supernatant is filtered through HSA Sepharose.

In the two cases, the immobilized proteins are eluted with 0.5 M acetic acid pH 2.8 and collected in 1 ml fractions.

The fractions collected are then analyzed on SDS-PAGE electrophoresis gel and by Immuno blot.

RESULTS

The cloning of the gene was carried out in three stages according to the strategy presented in Figure 4.

In a first stage, we confirmed the published part of the sequence with the exception of a T in the place of an A in position 103.

Then we determined the 3'-sequence of the gene and finally the 5'-sequence.

The entire gene was obtained by fusion of the two parts 8/4 and 3/14 and then cloned in the vector pRIT 28. The sequence corresponds to SEQ ID No. 13.

The protein is expressed in the form BBP40.

It is essentially obtained starting from inclusion bodies. For a 200 ml culture, fifteen milligrams of protein are purified.

The electrophoretic profile shows that BBP40, obtained after denaturation, is of high purity. The apparent molecular weight corresponds to the calculated theoretical weight which is 63 kDa.

The Immuno blot characterization shows that the purified protein is indeed recognized by a rabbit anti-P40 serum.

Example 5: Coupling of the p40 protein to the G_1A peptide

p40 (5 mg/ml, 40 mg) is dialyzed against 300 volumes of 0.1 M sodium phosphate buffer pH 7, zwittergent 3-14, 0.1%.

The dialyzate is adjusted to a concentration of 2 mg/ml with the aid of a 0.1 M carbonate buffer pH 9; zwittergent 3-14, 0.1%. Sodium dodecyl sulfate (SDS) is added to obtain a final concentration of 4%.

The G_1 peptide (10 mg/10 ml of 0.1 M carbonate buffer pH 9; 0.1% zwittergent 3-14) is added to the p40 solution. The pH is checked (between pH 9 and pH 10).

Add 220 µl of glutaraldehyde (2.5% in water) and stir for 24 hours at 4°C.

Add 5 ml of 0.1 M carbonate buffer pH 9; 0.1% zwittergent 3-14; check the pH (between pH 9 and pH 10); stir for 72 hours at 4°C.

Add 220 µl of glutaraldehyde (2.5% in water), check the pH, stir for 24 hours at +4°C.

The reaction is stopped by addition of 100 µl of 1 M lysine. The solution is dialyzed for 24 hours at 4°C.

The SDS is eliminated by double KCl precipitation.

The solution containing the p40 conjugate is frozen and used as such or lyophylized.

Example 6: Activity

Material and methods

C57BL/6 mice (N=5) are immunized on day 0, day 10 and day 20 by the subcutaneous route with 10 µg of G1, optionally coupled to a carrier, in the presence or absence of an adjuvant. The serum is collected and tested by ELISA. The anti-G1 or anti-carrier Igs are isolated on a BSA-G1 support and on a "carrier" support (KLH or TT or P40). The Igs are visualized with the aid of an anti-Ig rabbit peroxidase conjugate. The optical density is read at 450 nm and the anti-G1 antibody titer is given by the reciprocal of the last dilution giving twice the background noise. The results represent the mean ± standard deviation of the titers of 5 mice.

RESULTS

Induction of an immune response against G1A

The mice are immunized with G1A in different forms according to an identical immunization scheme. The antibody responses induced by the different forms of G1A are compared 28 days after the start of the experiment.

The synthetic G1A peptide administered pure does not induce any immune response even if it is coadministered with Freund's adjuvant. Presented with the carrier KLH, G1A induces a weak response which is significantly increased by the coadministration of Freund's adjuvant (FA). Presented with p40, G1A induces a greater response than that obtained in the conventional KLH/G1+AF, p40 immunization scheme to "self-adjuvant carrier" properties.

The results are presented in Figure 1.

Kinetics of the immune response to G1A

The mice are immunized with G1A in different

forms according to an identical immunization scheme. The antibody responses induced by the different forms of G1A are compared at the times: 7, 17, 28, 35, 42 days after the start of the experiment.

The anti-G1A response is significantly higher and more rapid when the mice are immunized with p40/G1A than the more conventional TT/G1A and KLH/G1A+AF immunizations. A single injection of p40/G1A allows an anti-G1A antibody titer of 1000 to be obtained in 7 days. This titer is obtained with TT/G1A or KLH/G1A+AF in 28 days. The maximum response (titer = 1/380 000), obtained after three injections, in 28 days is approximately 30 times greater than that obtained with KLH/G1A+AF and 70 times greater than that obtained with TT/G1A. The anti-G1A antibody titer holds steady without weakening until day 42.

The results are presented in Figure 2.

Kinetics of the immune response to the carrier

The mice are immunized with G1A coupled to a carrier according to an identical immunization scheme. The antibody responses induced by the different carriers are compared at the times 7, 17, 28, 35 and 42 days after the start of the experiment.

The anti-p40 response (titer close to 10,000) is higher than the anti-KLH response but not significantly different to the anti-TT response.

The results are presented in Figure 3.

CONCLUSION

The chemical coupling of the G1A peptide to the p40 protein allowed a significantly more important and more rapid anti-G1A response to be induced than that provoked by the KLH/G1A+AF or TT/G1A reference models. Coupling of the G1B peptide ought to induce similar responses.

Example 7: Evaluation of the protective potential of peptides and of recombinant proteins of glycoprotein G of respiratory syncytial virus (RSV) subgroup A coupled to p40 carrier protein

BALB/c mice were immunized with the following different preparations:

1) G1A synthetic peptide coupled to KLH (keyhole limpet hemocyanin) = KLH.G1A.
2) G1A synthetic peptide coupled to p40 carrier protein = p40.G1A.
3) p40 control alone.
4) Recombinant protein produced in E. coli: BBG2AδC coupled to p40 carrier protein = p40.BBG2AδC.
5) G1A synthetic peptide coupled to tetanus toxin (TT) carrier protein = TT.G1A.
6) TT control alone.
7) BB control alone.
8) Long RSV control (subgroup A).

The mice received 3 intramuscular doses (200 µg/mouse) with aluminum hydroxide as adjuvant (used currently in man). The results of the protection tests as well as the immunological profile of the sera are found in Table 2.

The following preparations confer complete protection following challenge with long RSV (strain A): p40.G1A, p40.BBG2AδC, with respect to TT.G1A which also confers very good protection comparable to the peptide KLH.G1A. In the ELISA test, they all recognize RSV antigen with the highest titer for p40.G1A = 1/12800.

As for the neutralization test, none of the preparations possess any neutralizing activity in vitro.

R combinant peptides and proteins	Protection		Elisa titer versus long RSV	Neutraliz-ation log 2/25 µl
	$DICT_{50}$ log10/g lungs challenge with long RSV (1.5×10^5/mouse) (subgroup A)			
	5 - 6 days	7 - 8 days		
KLH.G1A (100 to 157 µg)	2.45 2.45 ≤2.0±0.4 <1.7 p<0.001 <1.7 <1.7	2.45 2.15 ≤2.0±0.4 <1.7 p<0.001 <1.7 <1.7	4000	<3.0
P40.G1A (200 µg)	<1.7 <1.7 <1.7±0 <1.7 p<0.001 <1.7	<1.7 <1.7 <1.7±0 <1.7 p<0.001 <1.7	12800	<3.0
P40 controls (200 µg)	4.7 4.45 4.5±0.1 4.45 p<0.001 4.45	4.7 4.45 4.5±0.1 4.45 p<0.001 4.45	300	<3.0
P40.BBG2AδC (200 µg)	<1.7 <1.7 <1.7±0 <1.7 p<0.001 <1.7 <1.7	<1.7 <1.7 <1.7±0 <1.7 p<0.001 <1.7 <1.7	1700	<3.0

Table 2: Protection conferred and immunological profile of the sera after challenge with long RSV (A) following immunization of BALB/c mice with different recombinant proteins. (3-4 weeks after 3 doses i.m. with Aluminum hydroxide)

Recombinant peptides and proteins	Protection: $DICT_{50}$ log10/g lungs challenge with long RSV (1.5×10^5/mouse) (subgroup A)		Elisa titer versus long RSV	Neutralization log 2/25 µl
	5 - 6 days	7 - 8 days		
TT.G1A (200 µg)	<1.7 <1.7 <1.9±0.3 <1.7 p<0.001 <1.7 2.45	<1.7 <1.7 <1.9±0.3 <1.7 p<0.001 <1.7 2.45	7200	<3.0
TT controls (200 µg)	4.45 4.2 4.2±0.3 4.2 p=0.022 4.45 3.7	4.7 4.2 4.2±0.4 4.2 p=0.053 4.45 3.7	250	<3.0
BB controls (200 µg)	2.95 4.2 3.7±0.5 3.95 p=0.853 3.7 3.7	2.95 4.2 3.8±0.5 4.2 p=0.760 3.7 3.7	150	<3.0

Table 2 (continued): Protection conferred and immunological profile of the sera after challenge with long RSV (A) following immunization of BALB/c mice with different recombinant proteins. (3-4 weeks after 3 doses i.m. with Aluminum hydroxide)

Recombinant peptides and proteins	Protection		Elisa titer versus long RSV	Neutralization log 2/25 µl
	DICT$_{50}$ log10/g lungs challenge with long RSV (1.5×10^5/mouse) (subgroup A)			
	5 - 6 days	7 - 8 days		
Long RSV controls	<1.7 <1.7 <1.7±0 <1.7 p=0.001 <1.7 <1.7	<1.7 <1.7 <1.7±0 <1.7 p=0.001 <1.7 <1.7	76800	6.6
Controls, nonimmunized, challenged	3.95 3.95 3.7 3.7±0.2 3.45 3.95 3.45	3.95 4.2 3.7 3.8±0.3 3.45 3.95 3.7	150	<3.0
Controls, nonimmunized, unchallenged	No virus	No virus	150	<3.0

Table 2-(continued): Protection conferred and immunological profile of the sera after challenge with long RSV (A) following immunization of BALB/c mice with different recombinant proteins. (3-4 weeks after 3 doses i.m. with Aluminum hydroxide)

Example 8

Evaluation of the protective potential of peptides of glycoprotein G of respiratory syncytial virus (RSV) subgroup A and subgroup B coupled to KLH. Protection against a challenge carried out with the two subgroups of RSV.

BALB/c mice were immunized with the following different preparations:

1. C1A synthetic peptide coupled to KLH (keyhole limpet hemocyanin) = KLH-G1A
2. G1B synthetic peptide coupled to KLH (keyhole limpet hemocyanin) = KLH-G1B. The G1B peptide corresponds to the sequence G (174-187)δCys of the subgroup B whose sequence is:

Ser-Ile-Cys-Gly-Asn-Asn-Gln-Leu-Cys-Lys-Ser-Ile-Ser-Lys
|_____s-s_____|

3. KLH control
4. Long RSV control (subgroup A)
5. 8/60 VRS control (subgroup B)

The mice received 3 intramuscular doses (200 µg/mouse) with Freund's adjuvant. The results of the protection tests as well as the immunological profile of the sera are found in Table 3.

The preparation KLH-G1A allows complete protection against RSV subgroup A but not against RSV subgroup B. On the contrary, the preparation KLH-G1B allows complete protection against RSV subgroup B but not against RSV subgroup A. The ELISA test reflects the same situation.

Peptides coupled to KLH	PROTECTION $DICT_{10}$ log 10/g lungs		ELISA titer	
	Challenge Long RSV (subgroup A) 1.5×10^5/s (50/µl)	Challenge 8/60 RSV (subgroup B) 0.6×10^5/s (50/µl)	Versus long RSV (A)	Versus 8/60 RSV (B)
G1A	≤ 1.8 ± 0.3 n = 11 p < 0.001	3.3 ± 0.5 n = 10 p = 0.237	29 866	266
G1B	3.8 ± 0.8 n = 7 p = 0.517	≤ 2.1 ± 0.5 n = 8 p < 0.001	≤ 100	7 200
KLH control	3.7 ± 0.3 n = 11 p = 0.01	3.4 ± 0.3 n = 10 p = 0.6	≤ 200	133
VRS (A) control	≤ 1.7 ± 0 n = 11 p < 0.001	≤ 1.7 ± 0 n = 11 p < 0.001	≤ 68 266	51 200
VRS (B) control	≤ 1.7 ± 0 n = 10 p < 0.001	≤ 1.7 ± 0 n = 10 p < 0.001	≤ 76 800	68 266

Table 3: Protection conferred and immunological profile of the sera after challenge with long RS [sic] (subgroup A) or with RS [sic] 8/60 (subgroup B) following immunization of BALB/c mice with the peptides G1A and G1B.

Example 9: Veterinary application

Evaluation of th protective potential of G1vΔC peptide derived from protein G of the bovine strain of Respiratory Syncytial Virus (RSV) Lerch et al., 1990, J. Virol. 64:5559 coupled to KLH carrier protein.

174 187
Ser Thr Cys Glu Gly Asn Leu Ala Cys Leu Ser Leu Ser His having a disulfide bridge in position 176-182.

The peptide prepared by solid-phase synthesis using Boc chemistry is coupled to KLH using glutaraldehyde (Schaaper et al., Mol. Immunol. (1989) 26:81-85).

Two calves were immunized by the intramuscular route with 500 μg of G1vΔC-KLH with incomplete Freund's adjuvant 3 times at intervals of 3 weeks. One calf was immunized with KLH without G1VΔC peptide and with an incomplete Freund's adjuvant.

The animals are challenged with the Snook strain, 21 days after the last inoculation, by the intranasal and intratracheal route each with 1ml of virus titrating at 2 x 105/ml.

The virus titrated on calf kidney cells according to the plaque method is determined in nasopharyngeal washings 3 and 2 days respectively after challenge and 7 days in the lungs of the sacrificed animals.

CIRCULATING ANTIBODY RESPONSE:

Calf 3432 (KLH + FIA):

Date	Treatment	log10 ELISA titer			
		Peptide + KLH	Peptide	KLH	BRSV (Snook)
23/11	Day 0 vaccination	< 1.0	< 1.0	< 1.0	< 1.5
14/12	Day 21 vaccination	< 1.0	< 1.0	3.0	< 1.5
04/01	Day 42 vaccination	< 1.0	< 1.0	4.7	< 1.5
01/02	Day 70 VRS IN/IT	< 1.0	< 1.0	5.7	< 1.5
08/02	Day 77 sacrifice	1.5	< 1.0	4.8	< 1.5

Calf 3440 (Peptide - KLH + FIA)

Date	Treatment	log10 ELISA titer			
		Peptide + KLH	Peptide	KLH	BRSV (Snook)
23/11	Day 0 vaccination	< 1.0	< 1.0	< 1.0	< 1.5
14/12	Day 21 vaccination	1.6	< 1.0	< 1.0	< 1.5
04/01	Day 42 vaccination	3.8	2.6	1.7	1.9
01/02	Day 70 VRS IN/IT	2.7	2.8	2.6	3.7
08/02	Day 77 sacrifice	4.1	2.6	1.7	3.1

Calves to which 500 µg of G1vΔC - KLHen incomplete Freund's adjuvant was [sic] administered on three occasions at 3 week intervals.

RESPONSE TO THE VIRUS CHALLENGE

Calves	Vaccination	Nasopharyngeal widening		Day 7 pulmonary virus		% pneumoniae
		No. of days	max. titer	LBA titer (pfu/-ml)	Lung hom-og.	
3432	KLH+FIA	3	$5.1x_{-}0^{3}$	$1.4x10^{2}$	3/3	12
3440	peptide - KLH+FIA	2	$5.5x10^{2}$	<0.7	0/3	<1

CIRCULATING ANTIBODY RESPONSE

Calves	Vaccination	log10 ELISA titer (Snook BRSV)				
		Day 0	Day 24	Day 42	Day 68	Day 75
4138 4140	KLH + FIA *Peptide - KLH + FIA	< 1.5 <1.5	< 1.5 < 1.5	< 1.5 3.0	< 1.5 2.5 ≈	2.4 2.9

* Calf to which 500 µg of BP 4006 - KLH in incomplete Freund's adjuvant was ~~[sic]~~ administered on three occasions at three week intervals.

RESPONSE TO THE VIRUS CHALLENGE

Calves	Vaccination	Nasopharyngeal widening		Day 7 pulmonary virus		% pneumoniae
		No. of days	max. titer	LBA titer (pfu/-ml)	Lung homog.	
4138 4140	KLH+FIA peptide - KLH+FIA	5 4	4×10^1 2×10^3	6.5×10^2 7.0×10^1	2/3 3/3	27 2